COLD HARDINESS IN THE JAPANESE BEETLE, *POPILLIA JAPONICA* NEWMAN.

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Cold hardiness, or the ability of an organism to withstand low temperature may be considered from two points of view, (1) cold hardiness to the intensity factor or the ability to survive extreme low temperatures, and (2) cold hardiness to the quantity factor or ability to withstand long periods of low temperature. By low temperature is meant, temperature below that required for normal development.

The Japanese beetle, which was introduced into the United States about 1916, can be secured in large numbers, thus making intensive study possible. This insect represents a type of ecological group, the soil dwelling insects. It passes the winter in the larval stage; about 97 per cent. in the third instar; about 3 per cent. in the second. Cold hardiness to both the quantity and intensity factor of low temperature was studied. Both external and internal factors are involved in cold hardiness. These include such environmental factors as relative humidity and temperature, and such physiological conditions as nutritional state, health, blood conductivity and metabolic rate. Most of the work was done on larvæ. Some studies were made on adults and a few observations were made on cold hardiness in pupæ.

Methods and Apparatus.

Respiratory rate and quotient were determined by the modified Krogh manometer of Bodine and Orr (1925). Conductivity of blood and body fluids was determined by the ionometer, described by Gram and Cullen (1923). pH was determined with the type K potentiometer, using a small vessel capable of testing the pH of a drop. By this method several readings could be taken on the same larva. This method was described by Bodine and Fink

(1925). Occasionally a larva was found that would not bleed freely enough to give sufficient blood for a reading. Blood was usually taken from one of the feet. Relative humidity was maintained by pulling air over different concentrations of sulfuric acid by means of a suction pump.

Cold Hardiness to the Intensity Factor of Low Temperature.

In comparison with the oak borers previously studied by the author, Payne (1926), the Japanese beetles are less cold hardy and also exhibit less variation to low temperature. In Pennsylvania the most cold hardy Japanese beetle withstood — 28° C.; the most cold hardy oak borer — 47° C. The most cold hardy Japanese beetle collected in the field thus far withstood — 15° C.

Periodicity in cold hardiness to the intensity factor of cold is not as marked in the Japanese beetle as in the oak borers *Synchroa punctata* and *Dendroides canadensis*. Comparison of the three species in question tested at the same dates is shown in Table I.

Conditions other than seasonal which modify the cold hardiness of the Japanese beetle to the intensity factor of low temperature are (1) degree of dehydration, (2) disease incidence, (3) nutritional state, and (4) temperature at which the larvæ were kept. Although these larvæ are seldom collected in dry places normally, they are able to withstand a high degree of dehydration. Larvæ dried down to a pulpy condition in which the free water is reduced to a minimum are cold hardy to both intensity and quantity factors of low temperature. Severe dehydration is accompanied by a high death rate. Larvae can be dried down to one third of their body weight. In the dehydrated condition the Japanese beetle larvæ reach their greatest cold hardiness. Since eighty per cent. of dehydrated larvæ die the effect of dehydration may be considered highly selective, killing off those larvæ unable to hold water. Those larvæ capable of resisting dehydration are cold hardy. Relative humidity affects cold hardiness in a decided manner. The results of a series of different experiments with varying relative humidities is shown in Table II.

TABLE I.

Species.	September 29, 1926.			November 5, 1926.			December 11, 1926.		
	Mean Under-cooling.	σ.	C.V.	Mean Under-cooling.	σ.	C.V.	Mean Under-cooling.	σ.	C.V.
Synchroa punctata	−2.75	± .153	.056	− 7.5	±2.794	.374 −	−12.4	±3.422	.276
Dendroides canadensis	−8.45	±2.64	.313	−15.6	±4.087	.264 −	−17.34	±3.606	.208
Popillia japonica	−2.2	± .114	.0518	− 4.9	± .2156	.044	− 6.2	± .335	.054

Table II.

Conductivity of Blood of Japanese Beetle Larvæ Kept at Different Temperatures.

(Conductivity shown in % NaCl equivalent uncorrected for protein.)

0° C.	10° C.	20–22° C.	25° C.
.65	.6	.38	.33
.68	.61	.45	.35
.72	.604	.42	.38
.64	.58	.41	.39
.70	.604	.42	.40
.69	.55	.41	.41
.67	.58	.45	.375
.66	.6	.44	.39
.68	.6	.435	.40
.71		.46	.42
		.45	.39
		.43	.41
		.445	

Starvation at high temperatures, 20° C. or above, is fatal to the larvæ unless the relative humidity is kept high. When kept at high humidity, larvæ are able to withstand comparatively long periods of starvation. One hundred larvæ were kept without food for the month of May, 1927, but under conditions of 100 per cent. relative humidity or saturation. Each larva was placed in an individual vial and weighed before and after the starvation period. During the process they lost about one half of their body weight. None of them survived freezing, the lowest freezing point was — 1.7°C.; the highest — .65° C. Larvæ kept at + 10°C., or below their developmental temperature, lost one half of their body weight. Starvation conditions were assured by keeping the larvæ in sterile white sand kept moist with distilled water. About one fourth of these larvæ survived freezing. Changes in body weight under different conditions of starvation and dehydration are shown in Fig. 1. The effect of prolonged exposure to low temperature as well as starvation was involved in the experiment described above. The effect of different temperatures on cold hardiness as measured by blood conductivity is shown in Table III. Larvæ starved for one week at + 20° C. increased in cold hardiness. In general early stages of starvation are marked by an increase in cold hardiness, later

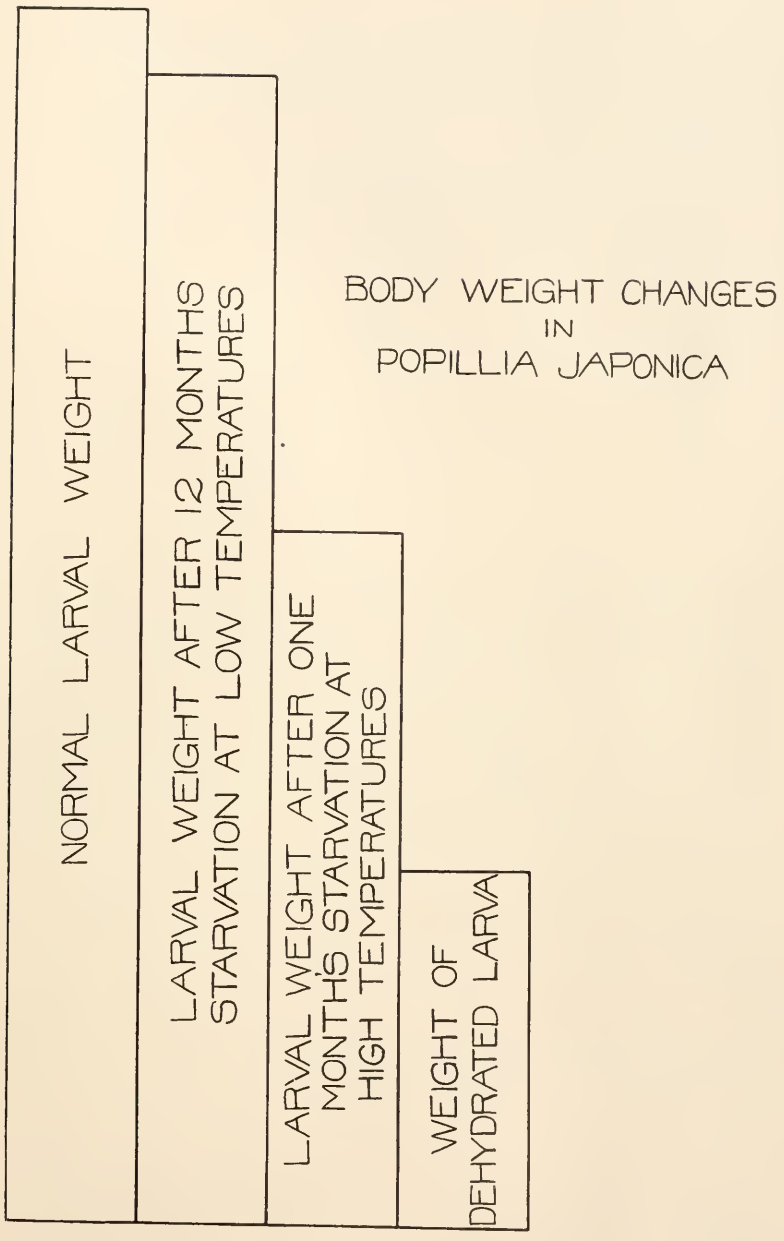
BODY WEIGHT CHANGES
IN
POPILLIA JAPONICA
NORMAL LARVAL WEIGHT
LARVAL WEIGHT AFTER 12 MONTHS
STARVATION AT LOW TEMPERATURES
LARVAL WEIGHT AFTER ONE
MONTH'S STARVATION AT
HIGH TEMPERATURES
WEIGHT OF
DEHYDRATED LARVA

FIG. 1.

Table III.

Effect of Different Relative Humidities on Conductivity at Temperature of 22° C.

(Conductivity shown in % NaCl equivalent uncorrected for protein.)

Saturation.	80%.	50%.
.33	.46	.72
.38	.45	.75
.35	.47	.76
.39	.465	.78
.40	.44	.73
.41	.455	.77
.375	.46	.74
.39	.44	.76
.40	.455	.77
.375	.46	.76
.38	.45	.82
.32	.46	.77
	.445	.78
	.43	

stages by a decrease. The point of decrease in cold hardiness from starvation comes when the digestive tract clears. In connection with this observation it is interesting to note that freshly molted larvæ are unable to withstand freezing until they have eaten. Pre-pupæ with clear digestive tracts are not cold hardy.

The occurrence of wilt disease in many of the specimens collected in the field offered an opportunity for the study of the effect of this disease on cold hardiness. Larvæ were collected at the same date and subjected to the same conditions of temperature and relative humidity, only healthy larvæ were studied. No larva showing typical symptoms of wilt disease or polyhedral-skränkheit was able to survive freezing. Since thermocouples used in diseased larvæ were difficult to sterilize and might infect healthy larvæ, cold hardiness was studied by measuring blood conductivity rather than freezing point depression. Conductivity decreases as the disease progresses. On the first day of apparent infection, conductivities of blood of diseased larvæ were below that of healthy larvæ. To produce such a marked change on the first day of infection, the causative organism must affect the blood very profoundly and very rapidly. On the other hand the change in conductivity may not be as rapid as it appears. The disease may be present in larvæ before it is detected by discol-

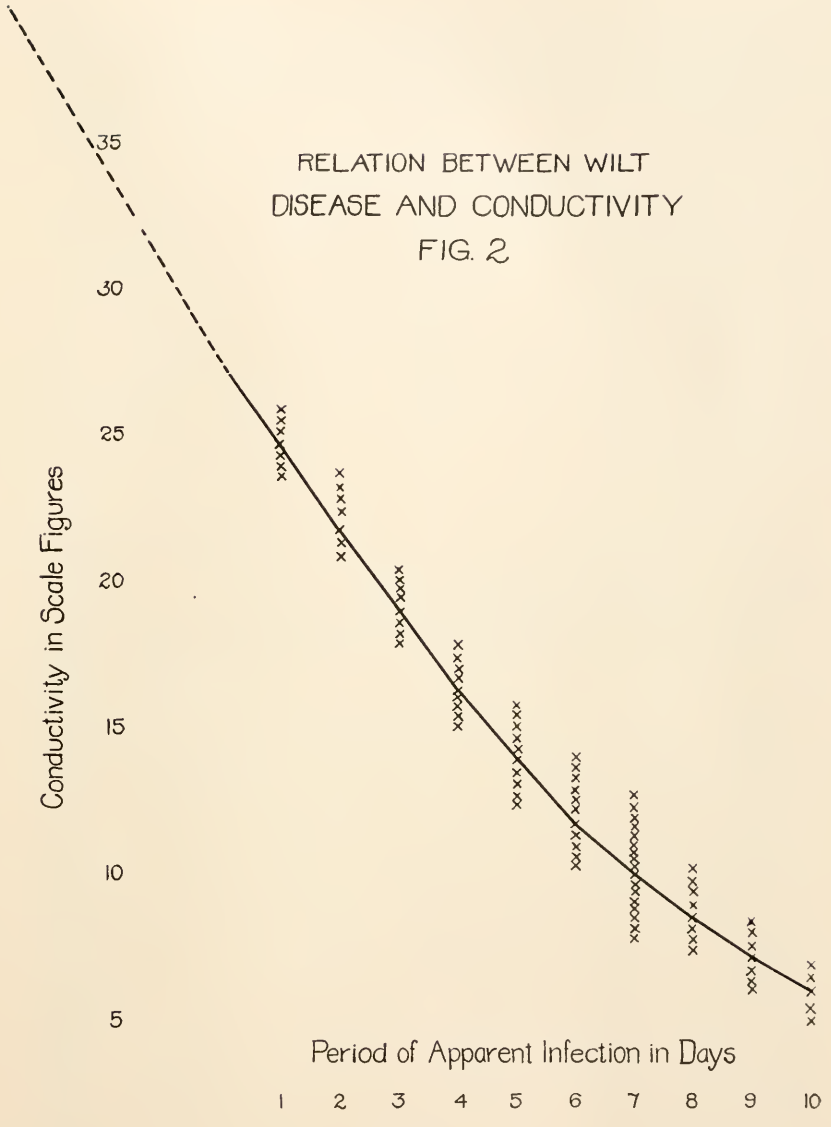

FIG. 2.

oration or wilting, and may be producing conductivity changes in the blood before other symptoms can be observed. A graph showing the relationship between day of apparent infection and blood conductivity is shown in Fig. 2. Table IV. shows the re-

Table IV.

Conductivity of Healthy and Diseased Japanese Beetle Larvæ.

(Conductivity shown in % NaCl equivalent uncorrected for protein.)

Wilt Disease.	Healthy.	Blackened by Freezing.
.21	.38	.6
.17	.4	.6
.10	.41	.604
.11	.4	.604
.04	.39	.58
.05	.375	.575
.07	.43	.61
.18	.425	.63
.12	.41	.62
.06	.39	.625
.13	.4	.64
.25		
.27		
.15		

sults of conductivity readings made on the blood of diseased larvæ in comparison with healthy ones.

Wilt disease is characterized by a pronounced blackening that precedes the final softening that occurs just before death. Blackening also has been observed when larvæ are frozen and thawed quickly. Blood from larvæ blackened after thawing always showed high conductivity. In these cases discoloration was believed to be due to changes in cell permeability releasing certain oxidative enzymes, which on escaping blackened the cells. The prothoracic segment is the first portion of the larvæ to discolor after freezing, both in the Japanese beetle and in the oak borers studied. Changes in permeability could be observed during the thawing process. Water apparently passes through the body wall where the chitin is thinnest. This water was frequently reabsorbed when the larvæ were kept under small bell jars. Larvæ losing water alone were generally able to survive freezing. When the fluid exhuding from the larva gave tests for amino-acids or proteins the larvæ always died. The exudate remained colorless for several days unless hydrogen peroxide was added, in which case it blackened quickly. Larvæ which showed the exudate after thawing were fixed and sectioned, but in these sections no gross differences from normal tissue could be detected. Broken

cell walls were not in evidence. The direct cause of death from extreme low temperature has been interpreted as due to an irreversible change in permeability rather than to a breaking of the cell walls.

If larvæ capable of surviving low temperature are ground up and filtered and the filtrate precipitated with lead acetate, there occurs in the filtrate an enzyme capable of breaking proteins down to amino acids at low temperatures and of building up proteins from amino acids at high temperatures. A similar enzyme has also been found in tussock moth eggs. Reversible reactions with proteases have been reported by Abderhalden (1914) from autolyzing tissues. Taylor (1909), found that a protein—"plastein"—could be formed from albuminose and proteolytic enzymes. The reversible reaction of starch to sugar at low temperatures and sugar to starch at high temperatures is a well known reaction that takes place in potato storage. The cold hardy mechanism of these larvæ studied may, in part, be due to enzyme action which transforms large protein molecules into smaller amino-acids. The larger number of osmotically active units thus formed would lower the freezing point.

Periodicity to cold hardiness is not as marked in the Japanese beetle as it is in some of the insects that are exposed to extremes of low temperature. Larvæ of the Japanese beetle live close enough to the surface of the ground to experience some seasonal change. During the spring and fall they are in addition subjected to diurnal temperature change. Cold hardiness in the larvæ appears to be closely related to their environment. These organisms are somewhat seasonal in their resistance to low temperatures. This periodic cold hardiness is shown in Table I. Comparison with oak borers and aquatic insects is shown more fully in a previous article by the author (Payne, 1926). Although the larva stage is the only one which overwinters in this climate, it was thought that studies on the cold hardiness of the adults would yield valuable material for the comparison of a stage exposed to winter conditions and a stage not normally exposed. Adults captured in summer and frozen without previous conditioning were able to survive ice formation within their tissues and to survive temperatures as low as — 20° C. Since it was im-

possible to obtain enough blood from the adults to make a conductivity reading none were made.

A beginning was made on the study of cold hardiness of the Japanese beetle pupæ. From present observations the age of the pupæ and consequently the degree of hydrolysis they are undergoing determines cold hardiness.

No changes in blood pH were found to be associated with cold hardiness in healthy larvæ. The pH obtained from a series of blood samples is shown in Table V. In the early stages of wilt

Table V.

Ph of Japanese Beetle Larvæ Blood Third Instar.

Each reading is an average of 3.

Healthy.	With Wilt Disease.
6.5	5.8
6.78	5.7
6.92	5.56
6.94	6.
6.5	5.91
7.16	5.82
7.18	5.83
6.66	5.97
6.77	6.1
7.1	5.84
7.17	5.92
7.	5.96
6.54	5.98
6.66	5.96
6.82	5.94
7.	5.9
6.35	5.84
6.51	5.97
7.1	5.95
7.1	

disease the pH was lower than in healthy larvæ. In the late stages of the disease the larvæ were in such condition that it was difficult to obtain blood by cutting off the feet.

The respiratory quotient tends to be high in both cold hardy and non-cold hardy specimens, ranging from .67 to .72. The respiratory quotient of starving larvæ tended to be higher than well fed larvæ regardless of the temperature at which they were kept. The respiratory rate in larvæ in which cold hardiness had

been induced was much lower than in the non-cold hardy individuals. Associated with the low respiratory rate of hibernating forms was the slight change in body weight occurring over a period of several months, as shown in Fig. 1.

Cold Hardiness to the Quantity Factor of Low Temperature.

Both the second and the third instars of the Japanese beetle larva are cold hardy to the quantity factor of low temperature except directly after molting or when the digestive tract is clear. Larvæ are markedly adapted to withstand long periods of low temperature. At the present writing there are still ten larvæ alive of one hundred which were placed at + 10° C. on December 6, 1925. These larvæ have now been kept over two years below their developmental temperature. Similar lots have been kept from six to twelve months at + 10° C. Graphs showing the number of larvæ surviving plotted against time in months in these experiments are shown in Fig. 3.

The relationship between survival for long periods at low temperatures and cold hardiness to the intensity factor of low temperature is shown in Table VI. The two types of cold hardi-

Table VI.

Survival after Freezing of Japanese Beetle Larvæ.

Kept at constant temperature of + 10° C. for varying periods of time.

Length of Time Kept at + 10° C.	Number Frozen.	Number Survived.	% Survived.
2 weeks	1,450	1,426	98.34
4 weeks	1,400	1,078	77
8 weeks	1,000	645	64.5
3 months	500	290	58
6 months	200	48	24

ness appear to be inversely related after a certain point has been reached. This decrease in cold hardiness to the intensity factor of low temperature cannot be interpreted as a simple loss in vitality since larvæ kept at low temperatures are able to complete their development when placed at room temperature with no higher death rate than larvæ maintained at room temperature.

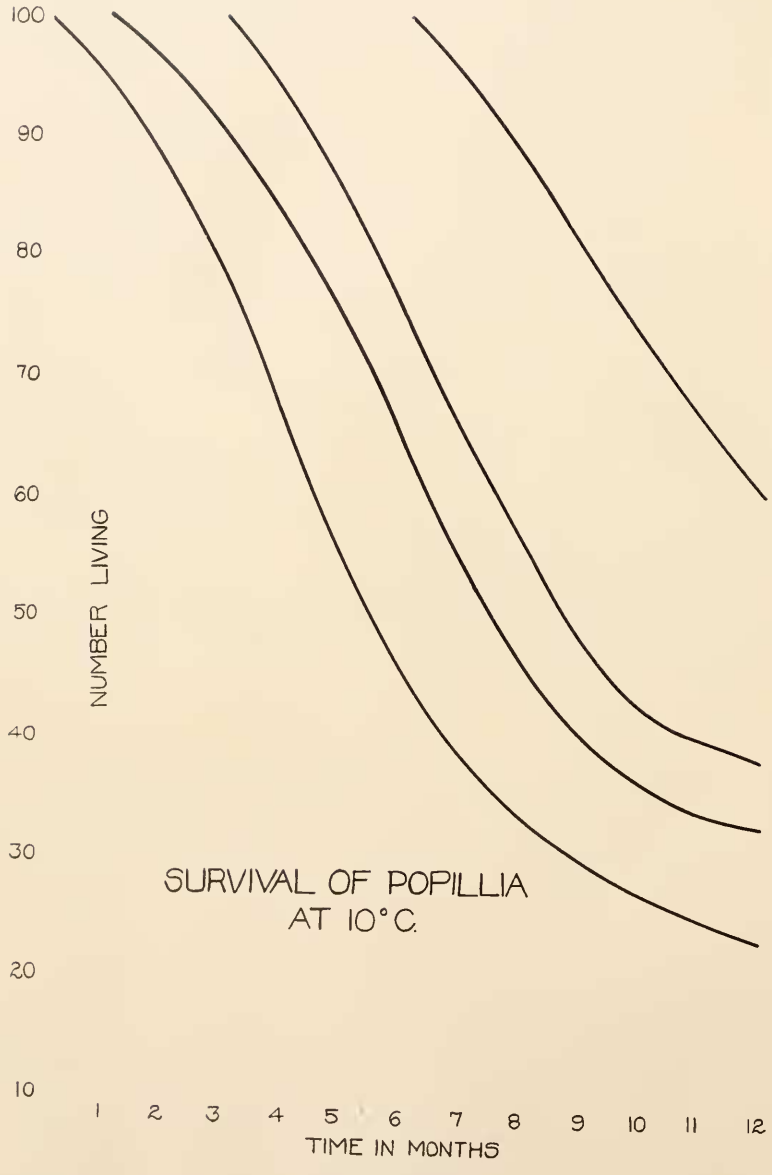
100
90
80
70
60
50
40
30
20
10
NUMBER LIVING
SURVIVAL OF POPILLIA
AT 10° C.
1 2 3 4 5 6 7 8 9 10 11 12
TIME IN MONTHS

FIG. 3.

Long periods of dormancy accelerate development when the larvæ kept at low temperatures are raised to developmental temperatures. Blood conductivity at first rises, then falls after two or more months when larvæ are placed at or below + 10° C.

The effect of rapid alternation between high and low temperatures on cold hardiness was tried with one hundred third instar larvæ. Temperatures of 0° C. and + 30° C. were alternated every twenty-four hours for one month. Neither of these temperatures is fatal. As controls one hundred larvæ were kept at 0° C. and one hundred at + 30° C. None died at + 30° C. Those alternated between + 30° C. and 0° C. died more rapidly than those kept at 0° C. Results of these experiments are shown in Fig. 4. In larvæ which had been exposed to wilt disease alternating temperature had no effect on length of life. None of these larvæ lived longer than ten days except when they were kept at or below 0° C. Healthy larvæ were considered exposed when they had been bitten by larvæ having wilt disease.

The respiratory quotient of larvæ cold hardy to the quantity factor of cold was somewhat variable but not connected to length of survival at low temperatures. In larvæ with clear digestive tracts it tended to become lower. In larvæ kept at + 10° C. it ranged from .69 to .73, or slightly higher than in larvæ cold hardy to the intensity factor of low temperature. In larvæ with clear digestive tracts low respiratory quotients were associated with lack of cold hardiness.

Low respiratory rate is associated with cold hardiness to the quantity factor of low temperature. Changes in body weight, as has been stated before, were very small with larvæ kept for long periods of time at + 10° C. These changes occurring in different states of nutrition and under varying temperature and humidity conditions are shown in Fig. 1.

Dehydration of larvæ is associated with cold hardiness to the quantity factor of low temperature as well as to the intensity factor of low temperature. Dehydration beyond two thirds of the body weight decreases cold hardiness to the quantity factor of low temperature. Over dehydrated larvæ lived but one day at 20° C. and not more than three days at + 10° C. or not more than four days at 0° C. Dehydrated larvæ have been kept for

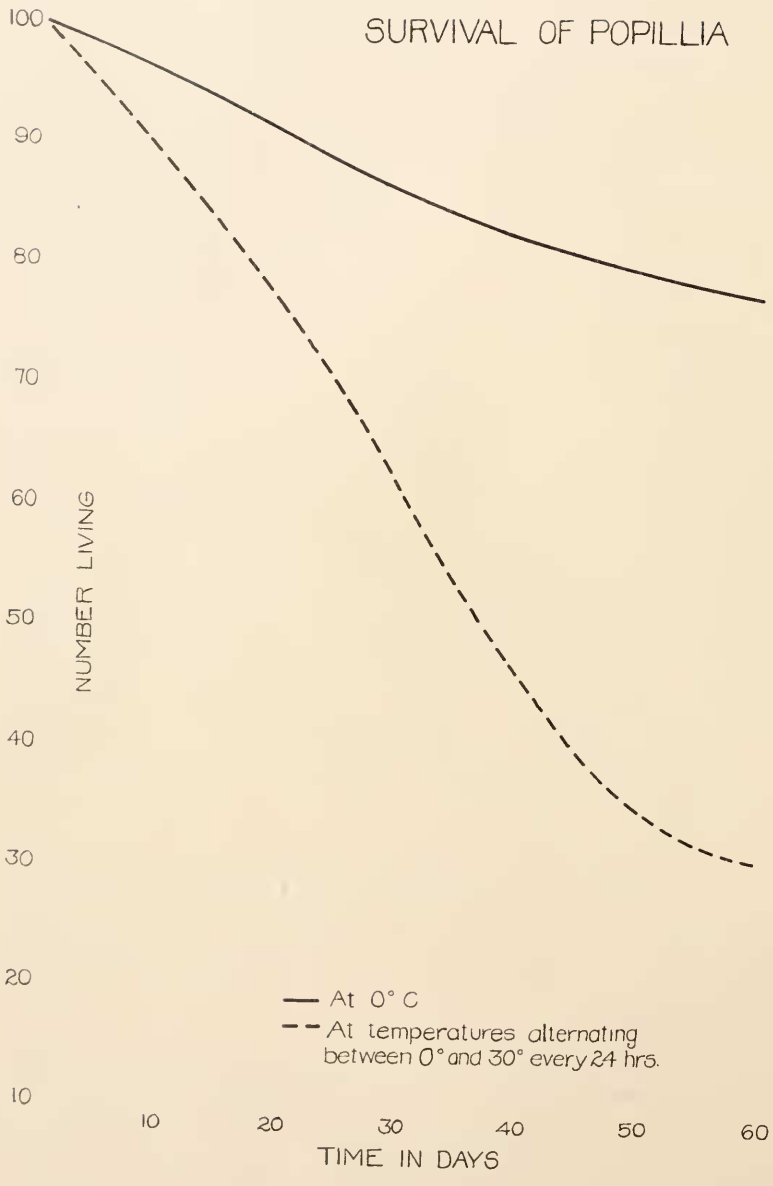
SURVIVAL OF POPILLIA
100
90
80
70
60
50
40
30
20
10
NUMBER LIVING
10
20
30
40
50
60
TIME IN DAYS
At 0° C
At temperatures alternating between 0° and 30° every 24 hrs.

FIG. 4.

one year at + 10° C. The experiment has not been continued long enough to determine whether or not dehydration increases the cold hardiness to the quantity factor of low temperature. Untreated larvæ are able to live two years or more below their developmental temperature. Dehydrated larvæ show very nearly the same death rate as undehydrated ones.

Literature.

Since the literature pertaining to cold hardiness has been recently brought together it seems hardly necessary to make a detailed list and discussion of it. Robinson (1927) has discussed and given experimental data on water binding capacity as a factor in cold hardiness. Robinson (1926), and the author (1926) have summarized the literature. Hibernation in regard to both its ecology and physiology has been recently treated by Fink (1925), Townsend (1926), and Holmquist (1926).

From a survey of the literature it would appear that no one factor is an adequate measure of cold hardiness. The development of a cold hardy from a non-cold hardy insect is a deep-seated physiological process which affects blood, and body fluids, respiratory rate and permeability. Nutritional state and environmental conditions also influence cold hardiness.

Acknowledgments.

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Summary.

1. Cold hardiness, both to the intensity factor and to the quantity factor of low temperature, were studied in the second and third instars of the Japanese beetle. Brief observations were made on pupæ and adults with regard to cold hardiness.

2. Japanese beetle larvæ are somewhat periodic in their cold hardiness to the intensity factor of low temperature, less so than the oak borers previously studied and more so than the aquatic insects.

3. Disease incidence, nutritional state, and degree of dehydration are associated with cold hardiness to the intensity factor of low temperature.

4. Development of cold hardiness to the quantity factor of low temperature is associated with loss of cold hardiness to the intensity factor except in extremely dehydrated individuals.

5. Marked permeability changes associated with enzyme action occur at the vital temperature minimum.

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